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(54) Title: FUSION PROTEINS THAT SPECIFICALLY INHIBIT PROTEIN SYNTHESIS IN NEURONAL CELLS

(57) Abstract: This invention relates to compositions and methods for the specific inhibition of protein synthesis in neuronal cells leading to neuronal cell death. More specifically, the invention relates to hybrid protein molecules that show high specificity for, and increased cytotoxicity in, neuronal cells. Such hybrid molecules are useful in a variety of conditions where localized inhibition of neuronal cell function is desirable.

FUSION PROTEINS THAT SPECIFICALLY INHIBIT PROTEIN SYNTHESIS IN NEURONAL CELLS

Related Applications

This application claims priority under 35 USC §119(e) from U.S. Provisional Patent Application Serial No. 60/165,807, filed on November 16, 1999, entitled A DIPHTHERIA/TETANUS TOXIN-BASED FUSION PROTEIN THAT SPECIFICALLY INHIBITS PROTEIN SYNTHESIS IN NEURONAL CELLS. The contents of the provisional application are hereby expressly incorporated by reference.

Government Support

This work was funded in part by grant numbers R01 NS38679-01, 1P01NS31248-02 and 5F32HS10064 from the National Institutes of Health, and CA-60934 from the National Cancer Institute. Accordingly, the United States Government may have certain rights to this invention.

Field of the Invention

This invention relates to compositions and methods for the specific inhibition of protein synthesis in neuronal cells leading to neuronal cell death. More specifically, the invention relates to hybrid protein molecules that show high specificity for, and increased cytotoxicity in, neuronal cells. Such hybrid molecules are useful in a variety of conditions where localized inhibition of neuronal cell function is desirable.

Background of the Invention

Bacterial protein toxins are the most deadly naturally occurring substances (Middlebrook, 1989). Despite their lethality, bacterial toxins are gaining increasing attention as therapeutic agents in human disease. Treatment of certain neuromuscular disorders involving local muscle spasticity or dystonia (see, e.g., U.S. Patent nos. 5,721,215; 5,677,308; 5,562,907, 5,053,005, etc.) involves injection of a chemodenervating agent, currently a botulinum toxin preparation (BOTOX®, Allergan, Irvine, CA), directly into the muscle, using, for example, a fine gage teflon-coated needle under electromyographic control to aid the physician in locating the intended intramuscular locus of the injection. A sufficient dose of the toxin acts on striated muscle to block release of the acetylcholine neurotransmitter from the presynaptic membrane resulting in varying degrees of effective denervation of the muscle

in regions contacted by the toxin (i.e., causing local paralysis). This results in an increase in post-synaptic acetylcholinesterase activity and an increase in the population of acetylcholine receptors, effects which occur as a characteristic physiological response to denervation. After a period of days, the axon terminals develop sprouting, and over a period of several months, collateral motor axons establish new neuromuscular connections with the muscle fiber. As neuromuscular junctions are regenerated, full function of the muscle returns along with the spasmodic contractions or hyperstimulation symptomatic of the disease.

Botulinum toxin treatment usually requires an identification of the muscles responsible for the dystonic movement so that the toxin can be injected into the appropriate neck muscles. Accordingly, polymyographic investigation may be a prerequisite to isolate the problem muscle areas. Botulinum toxin treatment is also known to be associated with a number of side effects. Such side effects include transient fatigue, dysphagia, neck weakness, hoarseness and localized pain. In addition, many patients who preliminarily respond to botulinum toxin therapy subsequently become non-responsive to the treatment. Accordingly, for many patients the botulinum injections simply constitute a preface to neurosurgery. Such treatments are both painful, unnecessarily dangerous, and fail to provide any long term treatment of the condition.

Tetanus toxin, when administered systemically or intramuscularly to animals, is selectively taken up by motor neurons in the brainstem and spinal cord (Habermann et al., *Naunyn-Schmiedebergs Arch. Pharmacol.*, 1973, 276:327-340). Both tetanus toxin and botulinum toxin, have a common binary structure in which the heavy chain appears to mediate binding, and the light chain is responsible for most of the toxicity. The carboxyl 451 amino acid fragment of the heavy chain ("tetanus toxin fragment C" or "TTC") retains the neuronal binding and uptake properties of the holotoxin without the toxic domains (Bizzini et al., *J. Neurochem.*, 1977, 28: 529-542; Morris, et al., *J. Biol. Chem.*, 1980, 255:6071-6076; Weller et al., *Toxicon*, 1986, 24: 1055-1063). TTC has been chemically conjugated to large proteins to enhance their uptake into neurons in tissue culture (Dobrenis et al., *Proc. Natl. Acad. Sci. USA*, 1992, 89:2297-2301) and neurons in animal models (Bizzini et al., *Brain Res.*, 1980, 193:221-227; Beaude et al., *Biochem. J.*, 1980, 271: 87-91; Fishman et al., *J. Neurol. Sci.*, 1990, 98: 311-325).

Summary of the Invention

The invention provides compositions and methods for the specific inhibition of protein synthesis in neuronal cells leading to neuronal cell death. More specifically, the

invention provides hybrid protein molecules (and compositions comprising such molecules) that show high specificity for, and increased cytotoxicity in, neuronal cells. Such hybrid molecules are useful in a variety of conditions where localized inhibition of neuronal cell function is desirable.

We have discovered that a hybrid protein, based on a fusion of certain diphtheria toxin and tetanus toxin domain moieties, is neuronal cell-specific and, unexpectedly, highly cytotoxic to the targeted neuronal cells when compared to the native toxin molecules. According to this aspect of the invention, a hybrid protein is provided. The hybrid protein comprises the catalytic and membrane translocation domains (or active portions thereof) of diphtheria toxin joined by a covalent bond to the binding domain (or binding portion thereof), of a neuronal cell-binding ligand. The binding domain can selectively target the hybrid protein to neuronal cells. In certain embodiments, the binding domain that selectively targets the hybrid protein to neuronal cells is tetanus toxin. In preferred embodiments, the binding domain that selectively targets the hybrid protein to neuronal cells is tetanus toxin fragment C (TTC). In some embodiments, the catalytic domain moiety of the diphtheria toxin retains its cytotoxic activity following uptake of the hybrid protein into a neuronal cell. In preferred embodiments, the catalytic domain moiety is the native diphtheria toxin catalytic domain. In further embodiments, the catalytic domain moiety contains mutations that enhance or reduce (but not substantially eliminate) its cytotoxicity following uptake of the hybrid protein into a neuronal cell.

According to another aspect of the invention, another hybrid protein is provided. In this aspect, the hybrid protein comprises a binding domain to selectively target the hybrid protein to neuronal cells, which domain again preferably is tetanus toxin or a neuronal cell binding domain thereof. The hybrid protein also includes the catalytic and membrane translocation domains of diphtheria toxin; the catalytic domain moiety containing mutations that abolish its cytotoxicity following uptake of the hybrid protein into a neuronal cell. The hybrid protein of this aspect, further comprises a cytotoxic moiety, other that the diphtheria catalytic domain moiety, joined to the mutated diphtheria catalytic domain moiety by at least one covalent bond. In any of the foregoing embodiments, the membrane translocation domain moiety (or membrane translocation portion thereof) of diphtheria toxin facilitates uptake of the hybrid protein into a neuronal cell.

According to yet another aspect of the invention, another hybrid protein is provided. The hybrid protein comprises the membrane translocation domain of diphtheria toxin joined by a covalent bond to the binding domain (or binding portion thereof), of a neuronal cell-

binding ligand, and a cytotoxic moiety, other than the diphtheria catalytic domain moiety, joined to the diphtheria membrane translocation domain moiety by at least one covalent bond. As in any of the foregoing embodiments of the invention, the binding domain can selectively target the hybrid protein to neuronal cells, and preferred binding domains are as described above.

According to a further aspect of the invention, a hybrid protein is provided. The hybrid protein comprises the membrane translocation domain of a protein joined by a covalent bond to the binding domain (or binding portion thereof), of a neuronal cell-binding ligand, and a cytotoxic moiety other than a diphtheria catalytic domain moiety, joined to the membrane translocation domain moiety of a protein by at least one covalent bond. As in any of the foregoing embodiments of the invention, the binding domain can selectively target the hybrid protein to neuronal cells, and preferred binding domains are as described above. In certain embodiments, the translocation domain moiety is the translocation domain moiety of a naturally occurring toxin. Preferred naturally occurring toxins include diphtheria toxin and Pseudomonas exotoxin A.

Another hybrid is a conjugate of any neuronal-specific binding ligand and any cytotoxic agent. Preferably the ligand is a nerve-cell binding fragment of tetanus toxin.

In other aspects of the invention, pharmaceutical compositions are provided. The pharmaceutical compositions comprise any of the foregoing neuronal cell-specific hybrid protein compositions of the invention, in a pharmaceutically effective amount to inhibit protein synthesis in a neuronal cell leading to neuronal cell death, and a pharmaceutically acceptable carrier.

According to another aspect of the invention, a method of inhibiting protein synthesis in a neuronal cell is provided. The method involves contacting a neuronal cell with any of the foregoing neuronal cell-specific hybrid proteins of the invention, in an effective amount to inhibit protein synthesis in the neuronal cell, leading to neuronal cell death.

According to still another aspect of the invention, a method of treating spasm or involuntary contraction in a muscle or a group of muscles in a subject, is provided. The method involves administering to a muscle or a group of muscles in a subject in need of such treatment, a hybrid protein composition of the invention, in an effective amount to enhance denervation of the muscle or group of muscles and inhibit spasm or involuntary contraction in the muscle or the group of muscles of the subject. In certain embodiments, the subject in need of such treatment has blepharospasm, strabismus, spasmodic torticollis, focal dystonia, jaw dystonia, occupational dystonia, corneal ulceration (protective ptosis), spasmodic

dysphonia (laryngeal dystonia), or facial dyskinesis. Facial dyskinesis includes Meige syndrome, hemifacial spasm, aberrant regeneration of facial nerves, and apraxia of eyelid opening. The administration in one embodiment is local administration.

According to yet another aspect of the invention, a method of enhancing relaxation or slackening of cutaneous tissue, is provided. The method involves locally administering to a cutaneous tissue (that includes subcutaneous administration) a hybrid protein composition of the invention, in an effective amount to enhance denervation of the muscle or group of muscles present subcutaneously to the cutaneous tissue to enhance relaxation or slackening of the cutaneous tissue. In certain embodiments, the hybrid protein composition of the invention is applied in an amount ranging from 0.0000001 to 20% by weight, based on the total weight of the composition. In some embodiments, the hybrid protein composition of the invention is applied in an amount ranging from 0.001 to 10% by weight, based on the total weight of the composition. In one embodiment, the method further comprises co-administering at least one of a hydroxy acid and a retinoid. In certain embodiments, the hydroxy acid is selected from the group consisting of α-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated. The retinoid can be retinoic acid, retinol or retinol esters.

According to another aspect of the invention, a method of lessening wrinkles or fine lines by relaxing or slackening cutaneous tissue, is provided. The method involves, locally administering to a cutaneous tissue (that includes subcutaneous administration) a hybrid protein composition of the invention, in an effective amount to enhance denervation of the muscle or group of muscles present subcutaneously to the cutaneous tissue to enhance relaxation or slackening of the cutaneous tissue, lessening wrinkles or fine lines. In certain embodiments, the hybrid protein composition of the invention is applied in an amount ranging from 0.0000001 to 20% by weight, based on the total weight of the composition. In some embodiments, the hybrid protein composition of the invention is applied in an amount ranging from 0.001 to 10% by weight, based on the total weight of the composition. In one embodiment, the method further comprises co-administering at least one of a hydroxy acid and a retinoid. In certain embodiments, the hydroxy acid is selected from the group consisting of α-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated. The retinoid can be retinoic acid, retinol or retinol esters.

According to another aspect of the invention, a method of controlling autonomic nerve function in a subject, is provided. The method involves locally administering to a target tissue or organ of a subject in need of such treatment a hybrid protein composition of the

invention, in an effective amount to enhance denervation in the target tissue or organ and control autonomic nerve function in the subject (as it relates to the targeted tissue or organ). In certain embodiments, the autonomic nerve function includes the function of an autonomic nerve which contributes to at least one symptom of rhinorrhea, otitis media, excessive salivation, asthma, COPD, excessive stomach acid secretion, spastic colitis or excessive sweating.

According to a further aspect of the invention, a method of treating a neurodegenerative disorder in a subject, is provided. The method involves locally administering to a target area in the brain of a subject in need of such treatment, a hybrid protein or pharmaceutical composition described in any of the foregoing aspects of the invention, in an effective amount to enhance neuronal cell-death in the target area of the brain of the subject. In certain embodiments, the subject has a neurodegenerative disorder selected from the group consisting of amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse Lewy body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral degeneration, torsion dystonia, familial tremor, Down's Syndrome, Gilles de la Tourette syndrome, Hallervorden-Spatz disease, diabetic peripheral neuropathy, dementia pugilistica, AIDS Dementia, age related dementia, age associated memory impairment, amyloidosis-related neurodegenerative diseases, traumatic brain injury, cerebral edema, schizophrenia, peripheral nerve damage, spinal cord injury, Wernicke-Korsakoff's related dementia. In important embodiments, the subject has Parkinson's disease.

According to a further aspect of the invention, methods for preparing medicaments useful in the treatment of neurodegenerative disorders, for controlling autonomic nerve function, for lessening wrinkles or fine lines by relaxing or slackening cutaneous tissue, for enhancing relaxation or slackening of cutaneous tissue, and/or for treating spasm or involuntary contraction in a muscle or a group of muscles, are provided.

These and other aspects of the invention, as well as various advantages and utilities, will be more apparent with reference to the detailed description of the preferred embodiments.

Brief Description of the Drawings

Figure 1. Graph showing a dose-response relationship of $DAB_{389}TTC$ cytotoxicity in primary neurons and N18-RE-105 cells.

Figure 2. Graph showing the comparative cytotoxicity of $DAB_{389}TTC$, DTx, and $DAB_{389}MSH$ in primary neuron cultures.

Figure 3. Bar graph showing inhibition of $DAB_{389}TTC$ cytotoxicity in primary neuron cultures by co-treatment with tetanus antitoxin.

Figure 4. Schematics depicting the secondary structure (Fig. 4A) and a linear format (Fig. 4B) of the diptheria toxin polypeptide.

Detailed Description of the Invention

We describe herein, compositions and methods for the specific inhibition of protein synthesis in neuronal cells leading to neuronal cell death. More specifically, the invention relates to hybrid protein molecules (and compositions comprising such molecules) that show high specificity for, and increased cytotoxicity in, neuronal cells. Such hybrid molecules are useful in a variety of conditions where localized inhibition of neuronal cell function is desirable.

We have discovered that a hybrid protein, based on a fusion of certain diphtheria toxin and tetanus toxin domains, is neuronal cell-specific and, unexpectedly, highly cytotoxic to the targeted neuronal cells when compared to the native toxin molecules. Because such compositions are specifically targeting and killing neuronal cells instead of just blocking neurosynaptic transmissions (as is the case, for example, with administration of the toxin itself), denervation is more effective and longer lasting.

The invention in certain aspects embraces hybrid proteins. According to one aspect, a hybrid protein comprises the catalytic and membrane translocation domains (or active portions thereof) of diphtheria toxin joined by a covalent bond to the binding domain (or binding portion thereof), of a neuronal cell-binding ligand.

The natural diphtheria toxin molecule consists of several functional "domains" which can be characterized, starting at the amino terminal end of the molecule, as a hydrophobic leader signal sequences (amino acids Val_{25} -Ala_1); enzymatically-active (catalytic domain) active Fragment A (amino acids Gly_1 -Arg_{193}); the proteolytically-sensitive disulfide loop l_1 (amino acids Cys_{186} -Cys_{201}), containing a cleavage domain; and Fragment B (amino acids Ser_{94} -Ser_{535}), which includes a translocation domain and a generalized binding domain flanking a second disulfide loop (l_2, amino acids Cys_{461} -Cys_{471}) (see Figures 4A and 4B).

The process by which diphtheria toxin intoxicates sensitive eukaryotic cells involves at least the following steps: (i) the binding domain of diphtheria toxin binds to specific

receptors on the surface of a sensitive cell; (ii) while bound to its receptor, the toxin molecule is internalized into an endocytic vesicle; (iii) either prior to internalization, or within the endocytic vesicle, the toxin molecule undergoes a proteolytic cleavage in l_1 between fragments A and B; (iv) as the pH of the endocytic vesicle decreases to below 6, the toxin spontaneously inserts into the endosomal membrane; (v) once embedded in the membrane, the translocation domain of the toxin facilitates the delivery of Fragment A into the cytosol; (vi) the catalytic activity of Fragment A (i.e., the nicotinamide adenine dinucleotide--dependent adenosine diphosphate (ADP) ribosylation of the eukaryotic protein synthesis factor termed "Elongation Factor 2") causes the death of the targeted cell. It is apparent that a single molecule of Fragment A introduced into the cytosol is sufficient to shut down the cell's protein synthesis machinery and kill the cell. The mechanism of cell killing by Pseudomonas exotoxin A, and possibly by certain other naturally-occurring toxins, is very similar.

"Translocation" as used herein, refers to the facilitation of movement of a chemical entity from the exterior surface of a cellular membrane (or what constituted the exterior surface prior to formation of an endocytic vesicle), through the membrane, and into the cytosol at the interior of the cell. A "translocation domain" is a protein or peptide which can translocate itself or molecule attached to it through a membrane. Such domains include segments of known proteins which, when the protein is bound to the exterior surface of a cellular membrane, is capable of translocating some portion of that protein through the membrane. A preferred translocation domain according to this aspect of the invention is fragment B of the diphtheria toxin or a functionally equivalent fragment variant or mimetic thereof.

In some embodiments, the catalytic domain moiety of the diphtheria toxin is included in the hybrid protein. It retains its cytotoxic activity following uptake of the hybrid protein into a neuronal cell. In preferred embodiments, the catalytic domain moiety is the native diphtheria toxin catalytic domain. In further embodiments, the catalytic domain moiety contains mutations that enhance or reduce (but not substantially eliminate) its cytotoxicity following uptake of the hybrid protein into a neuronal cell. Mutations that enhance or reduce the catalytic domain's cytotoxicity following uptake of the hybrid protein into a neuronal cell can be generated utilizing methods well known in the art, and examples of mutants with reduced cytotoxicity are described by Madhus et al., *Infection and Immunity*, 1992, 60(80):3296-3302. Given the teachings of Madhus, *infra*, and those of the present invention (e.g., cytotoxicity assays for preferred target cells and hybrid protein molecules according to the invention, Examples section), one of ordinary skill in the art can generate and screen a

number of such mutants using standard mutagenesis techniques. See, e.g., U.S. Patent No. 5,580,723 and Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, 1989. Other similar methods for creating and testing diphtheria toxin Fragment A (and other) cytotoxic mutants will be apparent to one of ordinary skill in the art.

The binding domain is any ligand that can selectively target the hybrid protein to neuronal cells. Such ligands are well known to those of ordinary skill in the art and include native ligands (such as neurotransmitters or other neuron specific binding agents) and nonnative targeting agents such as antibodies to neuronal cell surface antigens, fragments of antibodies, single chain antibodies, small binding peptides or other organic agents. Small binding peptides or other organic agents may be rationally designed as antibody mimetics or may be prepared using libraries of molecules and conventional screening technology. Such molecules also may be prepared as variants or modifications of known binding molecules such as variants or modifications of the nerve cell binding domain of tetanus toxin. In certain embodiments, the binding domain that selectively targets the hybrid protein to neuronal cells is tetanus toxin. In preferred embodiments, the binding domain that selectively targets the hybrid protein to neuronal cells is tetanus toxin fragment C (TTC) (see also Examples).

The preferred method for obtaining the hybrid protein molecules of this invention is by recombinant production, which involves genetic transformation of a host cell with a recombinant DNA vector encoding the hybrid proteins, expression of the recombinant DNA in the transformed host cell, and collection and purification of the hybrid protein. Preferably, the host organism is unicellular. More preferably, the host organism is prokaryotic.

The nucleotide sequence encoding the hybrid protein(s) must be operatively linked to suitable expression control sequences, and is typically incorporated into an expression vector, using conventional recombinant DNA techniques. See generally, Sambrook *et al, Molecular Cloning--A Laboratory Manual*, Cold Spring Harbor Press (1989); Ausubel *et al.* (eds.) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc. (1994). The design and construction of suitable expression vectors for producing the hybrid protein molecules of the invention is within ordinary skill in the art and involves routine experimentation. The expression vector is selected to be compatible with the host organism. A wide variety of host/expression vector combinations can be employed for expressing the hybrid protein molecule encoding DNA. Numerous host cell lines, expression vectors, and expression vector components are commercially available. Compatible host/expression vector combinations can be readily selected by those of skill in the art. In a preferred embodiment of

the invention, the unicellular host organism is E. coli, and the expression vector is pBluescript K+(Stratagene, LaJolla, Calif.). (See also Examples section)

The complete amino acid sequence of tetanus holotoxin and the *C. tetani* DNA sequence that encodes it have been published (Eisel *et al.*, *EMBO J.*, 1986, 5:2495-2502; Fairweather *et al.*, *Nuc. Acids. Res.*, 1986, 14:7809-7812). In addition, the TTC portion of the sequence has been defined, and TTC has been cloned and expressed (Fairweather *et al.*, *J. Bacteriol.*, 1986, 165:21-27; Halpern *et al.*, *Infection and Immunity*, 1990, 58:1004-1009). Accordingly, a DNA clone encoding the TTC moiety of the hybrid protein of this invention can be obtained by one of ordinary skill in the art, using a publicly available strain of *C. tetani* (e.g., from the American Type Culture Collection), published sequence information (e.g., Eisel *et al.*, *supra*; Halpern et al., *supra*), and conventional recombinant DNA techniques. Additional methods and sources of reagents can be found elsewhere herein (see, e.g., Examples), and in U.S. Patent 5,965,406 to Murphy and U.S. Patent 5,780,024 to Brown *et al.*, the contents of which are expressly incorporated herein by reference in their entirety.

According to another aspect of the invention, another hybrid protein is provided. In this aspect, the hybrid protein comprises a binding domain to selectively target the hybrid protein to neuronal cells, which domain again preferably is tetanus toxin or a neuronal cell binding domain thereof. The hybrid protein also includes the catalytic and membrane translocation domains of diphtheria toxin; the catalytic domain moiety, however, contains mutations that substantially abolish its cytotoxicity following uptake of the hybrid protein into a neuronal cell. The hybrid protein of this aspect, further comprises a cytotoxic moiety, other that the diphtheria catalytic domain moiety, joined to the mutated diphtheria catalytic domain moiety by at least one covalent bond (such as a disulfide bond, a peptide bond that is specifically susceptible to endocytic proteases, or a thioether bond). The bond may be selected such that it is cleaved under the chemical and enzymatic conditions that are likely to exist within endocytic vesicles of the targeted cell (such bond being herein specifically termed a "cleavable bond").

According to yet another aspect of the invention, another hybrid protein is provided. The hybrid protein comprises the membrane translocation domain of diphtheria toxin joined by a covalent bond to the binding domain (or binding portion thereof), of a neuronal cell-binding ligand, and a cytotoxic moiety, other than the diphtheria catalytic domain moiety, joined to the diphtheria membrane translocation domain moiety by at least one covalent bond. "Cytotoxic moieties"other than the diphtheria catalytic domain moiety, include well known entities such as a protease, a nuclease, or a toxin such as cholera toxin, LT toxin, C3 toxin,

Shiga toxin, E.coli Shiga-like toxin, ricin toxin, pertussis toxin, tetanus toxin, or Pseudomonas exotoxin A, all resulting to varying degrees of cytotoxicity (i.e. cell death) when taken up by the neuronal cell.

According to a further aspect of the invention, a hybrid protein is provided. The hybrid protein comprises the membrane translocation domain of a protein joined by a covalent bond to the binding domain (or binding portion thereof), of a neuronal cell-binding ligand, and a cytotoxic moiety other than a diphtheria catalytic domain moiety, joined to the membrane translocation domain moiety of a protein by at least one covalent bond. As in any of the foregoing embodiments of the invention, the binding domain can selectively target the hybrid protein to neuronal cells, and preferred binding domains are as described above. In certain embodiments, the translocation domain moiety is the translocation domain moiety of a naturally occurring toxin. Preferred naturally occurring toxins include diphtheria toxin and Pseudomonas exotoxin A.

In other aspects of the invention, pharmaceutical compositions are provided. The pharmaceutical compositions comprise any of the foregoing neuronal cell-specific hybrid protein compositions of the invention, in a pharmaceutically effective amount to inhibit protein synthesis in a neuronal cell leading to neuronal cell death, and a pharmaceutically acceptable carrier. Pharmaceutical preparations are described extensively below.

The invention also embraces methods of treatment for conditions where neuronal cell death (or denervation) is desirable. A number of such conditions call for such treatment, and are similar to those disease states where botulinum toxin administration is currently being standard. Examples of such disease states and methods of administration are described in detail in U.S. Patent nos.: 5,298,019 to Borodic, 5,721,215 to Aoki *et al.*, 5,677,308 to Lerner, 5,766,605 to Sanders *et al.*, 5,869,068 to De Lacharriere *et al.*, 5,714,468 to Binder, 5,670,484 to Binder, and other patents in the patent families of the foregoing, the contents of which are expressly incorporated herein by reference in their entirety. Methods of treatment according to the present invention comprise the administration of hybrid protein molecules that are more effective and unique in their mode of action (i.e., killing of the neuron cell), when compared to the more 'temporary' mode of action (i.e., blocking of synaptic transmission) of existing therapies, including the foregoing botulinum toxin regiment. Additionally, because of the efficiency in the neuronal cell targeting by the hybrid molecules of the invention, lower doses, are believed, to be required to achieve similar desirable effects to those achieved by the compositions and methods of the prior art.

Another important embodiment of the invention, is the treatment of subjects with a neurodegenerative disorder. "Neurodegenerative disorder" is defined herein as a disorder in which progressive loss of neurons occurs either in the peripheral nervous system or in the central nervous system. Selective killing of neurons in such disorders may be desirable in order to inhibit an unwanted symptom of the disorder (e.g. tremor), and/or inhibit further neuron degeneration. Local administration of the agents of the invention are again preferred. One of ordinary skill in the art would be familiar with the target area in the brain of a subject affected by the neurodegenerative disorder and therefore in need of treatment according to the instant invention. Examples of neurodegenerative disorders include: (i) *chronic neurodegenerative diseases* such as familial and sporadic amyotrophic lateral sclerosis (FALS and ALS, respectively), familial and sporadic Parkinson's disease, Huntington's disease, familial and sporadic Alzheimer's disease, multiple sclerosis, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse Lewy body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral degeneration, torsion dystonia, familial tremor, Down's Syndrome, Gilles de la Tourette syndrome, Hallervorden-Spatz disease, diabetic peripheral neuropathy, dementia pugilistica, AIDS Dementia, age related dementia, age associated memory impairment, and amyloidosis-related neurodegenerative diseases such as those caused by the prion protein (PrP) which is associated with transmissible spongiform encephalopathy (Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker syndrome, scrapic, and kuru), and those caused by excess cystatin C accumulation (hereditary cystatin C angiopathy); and (ii) *acute neurodegenerative disorders* such as traumatic brain injury (e.g., surgery-related brain injury), cerebral edema, peripheral nerve damage, spinal cord injury, and Wernicke-Korsakoff's related dementia (alcohol induced dementia). The foregoing examples are not meant to be comprehensive but serve merely as an illustration of the term "neurodegenerative disorder."

Most of the *chronic neurodegenerative diseases* are typified by onset during the middle adult years and lead to rapid degeneration of specific subsets of neurons within the neural system, ultimately resulting in premature death.

Alzheimer's disease is the most important of the neurodegenerative diseases due to the high frequency of occurrence within the population and the fatal course of the disease. It is characterised by loss of function and death of nerve cells in several areas of the brain leading to loss of cognitive function such as memory and language. Two forms of the disease exist: presenile dementia, in which the symptoms emerge during middle age, and senile dementia

which occurs in the elderly. Both forms of the disease appear to have the same pathology. The cause of nerve cell death is unknown but the cells are recognised by the appearance of unusual helical protein filaments in the nerve cells (neurofibrillary tangles) and by degeneration in cortical regions of brain, especially frontal and temporal lobes. A clear genetic predisposition has been found for presenile dementia. Familial autosomal dominant cases have been reported and the majority of individuals with trisomy 21 (Down's syndrome) develop presenile dementia after the age of 40. The familial Alzheimer's cases map to chromosomes 14, 19 and 21, with more than one locus on 21.

Amyotrophic lateral sclerosis (ALS) is the most commonly diagnosed progressive motor neuron disease. The disease is characterized by degeneration of motor neurons in the cortex, brainstem and spinal cord (Principles of Internal Medicine, 1991 McGraw-Hill, Inc., New York; Tandan et al. Ann. Neurol, 18:271-280, 419-431, 1985). Generally, the onset is between the third and sixth decade, typically in the sixth decade. ALS is uniformly fatal, typically within five years (Kurland et al., Proc Staff Meet Mayo Clin, 32:449-462, 1957). The cause of the disease is unknown and ALS may only be diagnosed when the patient begins to experience asymmetric limb weakness and fatigue, localized fasciculation in the upper limbs and/or spasticity in the legs which typifies onset.

In ALS the neurons of the cerebral cortex and anterior horns of the spinal cord, together with their homologues in some of the motor nuclei of the brain stem, are affected. The class of neurons affected is highly specific: motor neurons for ocular motility and sphincteric motor neurons of the spinal cord remain unaffected until very late in the disease. Although death occasionally results shortly after the onset of the symptomatic disease, the disease generally ends with respiratory failure secondary to profound generalized and diaphragmatic weakness. About 10% of ALS cases are inherited as an autosomal dominant trait with high penetrance after the sixth decade (Mulder et al. Neurology, 36:511-517, 1986; Horton et al. Neurology, 26:460-464, 1976). In almost all instances, sporadic and autosomal dominant familial ALS (FALS) are clinically similar (Mulder et al. Neurology, 36:511-517, 1986; Swerts et al., Genet. Hum, 24:247-255, 1976; Huisquinet et al.. Genet. 18:109-115, 1980). It has been shown that in some but not all FALS pedigrees the disease is linked to a genetic defect on chromosome 21q (Siddique et al., New Engl. J. Med., 324:1381-1384, 1991.

Parkinson's disease (paralysis agitans) is a common neurodegenerative disorder which appears in mid to late life. Familial and sporadic cases occur, although familial cases account for only 1-2 percent of the observed cases. The neurological changes which cause this disease are somewhat variable and not fully understood. Patients frequently have nerve cell loss with

reactive gliosis and Lewy bodies in the substantia nigra and locus coeruleus of the brain stem. Similar changes are observed in the nucleus basalis of Meynert. As a class, the nigrostriatal dopaminergic neurons seem to be most affected. The disorder generally develops asymmetrically with tremors in one hand or leg and progresses into symmetrical loss of voluntary movement. Eventually, the patient becomes incapacitated by rigidity and tremors. In the advanced stages the disease is frequently accompanied by dementia. Diagnosis of both familial and sporadic cases of Parkinson's disease can only be made after the onset of the disease.

Huntington's disease is a progressive disease which is always transmitted as an autosomal dominant trait. Individuals are asymptomatic until the middle adult years, although some patients show symptoms as early as age 15. Once symptoms appear, the disease is characterized by choreoathetotic movements and progressive dementia until death occurs 15-20 years after the onset of the symptoms. Patients with Huntington's disease have progressive atrophy of the caudate nucleus and the structures of the basal ganglia. Atrophy of the caudate nucleus and the putamen is seen microscopically where there is an excessive loss of neural tissue. However, there are no morphologically distinctive cytopathological alterations which have been observed.

Although some of the characteristic mental depression and motor symptoms associated with Huntington's may be suppressed using tricyclic antidepressants and dopamine receptor antagonists, respectively, no therapy exists for slowing or preventing of the underlying disease process. Huntington's disease appears to map to a single locus on chromosome 4 and a linkage test currently exists for the clinical assessment of disease risk in presymptomatic individuals with afflicted relatives.

Hallervorden-Spatz disease is a neurodegenerative disease which affects the neurons in the region of the basal ganglia. The symptoms generally appear during childhood and adolescence and the disease appears with an inheritance pattern that appears to be autosomal recessive. Patients show abnormalities in muscle tone and movement such a choreoathetosis and dystonia similar to that seen in parkinsonism. As the disease progresses there is increasing dementia. Death generally occurs approximately ten years after onset. There is no known presymptomatic diagnosis, cure or treatment for Hallervorden-Spatz disease. However, iron toxicity has recently been implicated in the progression of this disease Greenfield, Neuropatholoay, W. Blackwood & J. A. N. Corsellis, Eds. (Edinborgh; T. and A. Constable, Ltd., 1976) pages 178-180. As a result of this implication, the chelating agent deferoxamine mesylate has been administered to patients. However, this therapeutic approach

has shown no definite benefit (Harrison's Principles of Internal Medicine, Wilson et al. Eds., McGraw-Hill, Inc., New York, 1991).

Olivopontocerebellar atrophy (OPCA) is a disease classification which includes a number of disorders characterized by a combination of cerebellar cortical degeneration, atrophy of the inferior olivary nuclei and degeneration and disappearance of the pontine nuclei in the basis pontis and middle cerebellar peduncles. Autosomal dominant inheritance is characteristic in most families. In one family, termed the Schut family, genetic linkage has been shown to chromosome 6. An excess of glutamate has been implicated as the causative agent in this disease. A gene with an expanded CAG trinucleotide repeat causes one form of OCPA has now been identified and eluted sequencing can be used for diagnosis (Orr et al., Nature Genetics 4:221-226, 1993).

The hybrid protein molecules of the invention are administered in effective amounts. An effective amount is a dosage of the hybrid protein molecule sufficient to provide a medically desirable result. The effective amount will vary with the particular condition being treated, the age and physical condition of the subject being treated, the severity of the condition, the duration of the treatment, the nature of the concurrent therapy (if any), the specific route of administration and like factors within the knowledge and expertise of the health practitioner. For example, in connection with treating spasm or involuntary contraction in a muscle or a group of muscles in a subject, an effective amount is that amount which inhibits or reduces the spasm or involuntary contraction. Likewise, an effective amount for lessening the appearance of wrinkles or fine lines would be an amount sufficient to lessen or inhibit the muscular contractile tone of the group of muscles present subcutaneously under the wrinkled cutaneous surface so as to allow relaxation of the cutaneous surface and enhance its smoothness. Thus, it will be understood that the hybrid protein molecules of the invention can be used to treat the above-noted conditions according to the preferred modes of administration described below. It is preferred generally that a maximum dose be used, that is, the highest safe dose according to sound medical judgment. Repeated administrations of small doses so as to reduce 'spillage' and therefore unnecessary toxicity in the non-affected, non-targeted tissue/neurons are also preferred.

A subject, as used herein, refers to any mammal (preferably a human, and including a non-human primate, cow, horse, pig, sheep, goat, dog, cat or rodent) with a condition requiring inhibition of protein synthesis in neuronal cells, leading to neuronal cell death and extended periods of denervation and/or paralysis (such as the conditions described above).

A hybrid protein molecule of the invention may be administered alone or as part of a pharmaceutical composition. Such a pharmaceutical composition may include the hybrid protein molecule in combination with any standard physiologically and/or pharmaceutically acceptable carriers which are known in the art. The compositions should be sterile and contain a therapeutically effective amount of the hybrid protein molecule in a unit of weight or volume suitable for administration to a patient. The term "pharmaceutically-acceptable carrier" as used herein means one or more compatible solid or liquid filler, diluents or encapsulating substances which are suitable for administration into a human or other animal. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy. Pharmaceutically acceptable further means a non-toxic material (to cells other than neuronal cells), that is compatible with a biological system such as a cell, cell culture, tissue, or organism. The characteristics of the carrier will depend on the route of administration. Physiologically and pharmaceutically acceptable carriers include diluents, fillers, salts, buffers, stabilizers, solubilizers, and other materials which are well known in the art.

Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils.

Compositions suitable for parenteral administration conveniently comprise sterile aqueous and non-aqueous preparations of the hybrid protein molecules of the invention. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate, and including synthetic mono- or di-glycerides. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally-acceptable diluent or solvent, for example, as a solution in 1,3-butane diol. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulations suitable for oral, subcutaneous, intravenous, intramuscular, etc. administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA. Preservatives and other additives may also be present such as, for example, antimicrobials, anti-oxidants, chelating agents, and inert gases and the like.

A variety of administration routes are available. The particular mode selected will depend, of course, upon the particular drug selected, the severity of the condition being treated, and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include rectal, topical, nasal, or parenteral routes. The term "parenteral" includes subcutaneous, intramuscular, intradermal or topical infusion. Intramuscular routes are preferred. Oral and intravenous administration should be avoided due to the toxicity associated with the agents of the invention.

The pharmaceutical compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the hybrid protein molecules of the invention into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the hybrid protein molecules into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

Compositions suitable for parenteral administration include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Other delivery systems can include time-release, delayed release or sustained release delivery systems. Such systems can avoid repeated administrations of the hybrid protein molecules of the invention, increasing convenience to the subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. They include the above-described polymeric systems, as well as polymer base systems such as poly(lactide-glycolide), copolyoxalates, polycaprolactones, polyesteramides, polyorthoesters, polyhydroxybutyric acid, and polyanhydrides. Microcapsules of the foregoing polymers containing drugs are described in, for example, U.S. Patent 5,075,109. Delivery systems also include non-polymer systems that are: lipids including sterols such as cholesterol, cholesterol esters and fatty acids or neutral fats such as mono- di- and tri-glycerides; hydrogel release systems; sylastic systems; peptide based systems; wax coatings; compressed tablets using conventional binders and excipients; partially fused implants; and the like. Specific examples include, but are not limited to: (a) erosional systems in which the Akt molecule is contained in a form within a matrix such as those described in U.S. Patent Nos. 4,452,775, 4,675,189, and 5,736,152, and (b) diffusional systems in which an active

component permeates at a controlled rate from a polymer such as described in U.S. Patent Nos. 3,854,480, 5,133,974 and 5,407,686. In addition, pump-based hardware delivery systems can be used, some of which are adapted for implantation.

Use of a long-term sustained release implant may be particularly suitable for treatment of chronic conditions. Long-term release, are used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 30 days, and preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

The hybrid protein molecules of the invention may be administered alone or in combination (co-administered) with the above-described drug therapies by any conventional route, including injection, repeated injection, topical application, etc., over time. The administration may, for example, be intraperitoneal, intramuscular, intra-cavity, subcutaneous, or transdermal. When using the hybrid protein molecules of the invention, direct administration to the affected site (e.g., muscles with involuntary spasm, wrinkle, etc.) such as administration by injection, is preferred.

The term "co-administered," means administered substantially simultaneously with another agent. By substantially simultaneously, it is meant that a hybrid protein molecule of the invention is administered to the subject close enough in time with the administration of the other agent (e.g., an anti-wrinkling agent, etc.). The other agent may be present in a different formulation than the hybrid protein molecule of the invention, or it may be part of the same formulation.

The co-administered agent can act cooperatively, additively or synergistically with a hybrid protein molecule of the invention to produce a desired effect, for example, lessening of wrinkles. The other agent is administered in effective amounts. Such amounts maybe less than these sufficient to provide a therapeutic benefit when the agent is administered alone and not in combination with a hybrid protein molecule of the invention. A person of ordinary skill in the art would be able to determine the effective amounts needed.

The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

Examples

Experimental procedures

Materials and Methods

All DNA modifying enzymes were purchased from well known commercial sources. A codon-engineered cDNA clone for TTC was obtained from Dr. Neil Fairweather at Imperial College, London, UK (*J. Bacteriology*, 1986, 165:21-27). A cDNA encoding for the catalytic and membrane translocation domains of DTx was generated essentially as described in U.S. Patent 5,965,406, issued October 12, 1999 to Murphy JR, entitled "Recombinant DNAs encoding three-part hybrid proteins," the contents of which are incorporated herein in their entirety. A diphtheria toxin-melanocyte stimulating hormone fusion toxin, $DAB_{389}MSH$, was provided by these investigators as well (Murphy et al., *Proc. Natl. Acad. Sci. USA*, 1986, **83**:8258-8261). Recombinant TTC was purchased from Boehringer Mannheim (Indianapolis, IN). Diphtheria toxin and rabbit anti-TTC antisera were purchased from Calbiochem (La Jolla, CA), while mouse monoclonal antibody against the catalytic domain of DTx was purchased from Accurate Chemical and Scientific (Westbury, NY). Additional immunochemical reagents were purchased from Vector Laboratories (Burlingame, CA). Chloroquine and monensin were from Sigma (St. Louis, MO).

Plasmid Constructs

A fusion gene encoding the first 388 amino acids of DTx linked to TTC was constructed by slight modification of the previously described plasmid, pET-JV127. Derived from the prokaryotic expression vector pET11d (Novagen; Madison, WI), pET-JV127 encodes for a DTx-interleukin 2 fusion toxin, $DAB_{389}IL-2$ (vanderSpek et al., *J. Biol. Chem.*, 1993, **268**:12077-82). The present DTx:TTC fusion protein, designated $DAB_{389}TTC$, was assembled by replacing the IL-2 cDNA in pET-JV127 with a 1.4 kb semisynthetic cDNA for TTC obtained from plasmid pMAL:TetC (Figueiredo et al., *Infect. Immun.* 1995, **63**:3218-3221; Makoff et al., *Nucleic Acids Res.* 1989, **17**:10191-10202). The amino terminus of TTC was fused to the carboxyl terminus of the DAB389 moiety because the normal position of the respective receptor binding domains for both DTx and TTx is at the toxin's carboxyl terminus. To allow ligation of the 5' end of the TTC cDNA to the 3' terminus of the DAB_{389} coding sequence in pET-JV127, a PCR protocol was first employed to introduce a new SphI restriction site just upstream from the beginning of the TTC coding sequence. This PCR reaction used a partially degenerate forward primer that encoded for an SphI site at the 5' end of the primer as well as a BamH I site immediately upstream from the SphI site to allow subcloning of the PCR product back into plasmid pMALc:TetC. The reverse primer was positioned over a unique SacII restriction site in the TTC cDNA located about 0.4 kb downstream from the start of the coding sequence. The resulting 0.4 kb PCR product was

then restricted with BamHI/SacII and inserted by fragment exchange back into pMAL:TetC. The sequence of the PCR product was subsequently checked by the dideoxy chain termination method (Sanger et al., *Proc. Natl. Acad. Sci. USA* 1977, **74**:5463-5467). Finally, the IL-2 cDNA in pET-JV127 was replaced with the modified TTC cDNA from pMAL:TetC using SphI/HindIII fragment exchange, creating plasmid pETIId:DAB_{389}TTC. A similar fusion construct containing the E149S mutation in the DTx catalytic domain (DA(E149S)B_{389}TTC) was assembled in parallel using the plasmid pET-JV127(E149S) (vanderSpek et al., *J. Biol. Chem.* 1996, **271**:12145-12149).

Fusion Protein Purification

The DAB_{389}TTC and DA(E149S)B_{389}TTC fusion proteins were expressed in *E. coli* strain BL21(DE3) (Novagen). Liquid broth cultures (1.5L) in NZCYM media (Sigma) were propagated to an $O.D._{600}$ of 0.5-0.6 in a shaking incubator at 37°C, at which point IPTG was added to a final concentration of 0.5 mM to induce target protein expression. Bacteria were harvested 4 hours later by centrifugation at 5000g for 10 minutes and then stored at -20°C until further use.

Fusion proteins were purified from total soluble bacterial protein using ammonium sulfate precipitation followed by ion exchange chromatography. After resuspending the bacterial pellet in 50 ml of cold 0.1M potassium phosphate buffer, pH 8.0 (containing 2 mM EDTA, 20 mM β-mercaptoethanol, 0.5% Triton X-100, and Complete Antiprotease Cocktail [Boehringer Mannheim]), bacteria were lysed by lysozyme treatment followed by sonication. The total lysate was centrifuged at 20,000g for 20 minutes to obtain the soluble protein fraction (supernatant), to which 25 grams of solid ammonium sulfate was slowly added with stirring. The precipitated protein was collected by centrifugation at 20,000g for 20 minutes and resolubilized in 20 ml of 0.05 M Tris-HCl , pH 8.4, containing 2 mM EDTA, 20 mM β-mercaptoethanol, and Complete antiprotease cocktail without EDTA. The sample was then dialyzed overnight at 4°C against 0.05 M Tris-HCl, pH 8.4, containing 0.2 mM phenylmethylsulfonyl fluoride.

DEAE anion exchange chromatography was carried out at by passing the dialyzed ammonium sulfate cut over a 6 ml bed volume of DEAE Sepharose Fast Flow (Amersham Pharmacia) at a flow rate of 0.6 ml/min. The column was then washed with 100 ml of 0.02 M Tris-HCl , pH 8.4, containing 100 mM NaCl and Complete Antiprotease Cocktail without EDTA. Sample remaining bound to the column was subsequently eluted using a step gradient of 125, 150, and 175 mM NaCl in 0.02 M Tris-HCl , pH 8.4. After sample purity was

assessed by Coomassie Blue staining of SDS-PAGE gels run under reducing conditions, fractions were pooled and buffer exchanged against phosphate-buffered saline using a Centricon Plus-20 (Amicon; 50,000 MW cutoff). Sample protein concentrations were determined by Pierce Coomassie Blue Assay. The antigenic identity of $DAB_{389}TTC$ and DA(E149S)B_{389}TTC fusion proteins was confirmed by immunoblot analysis using rabbit anti-TTC antisera (1:100,000 dilution) and mouse anti-DTx antibody (1:1000 dilution). Purified sample protein was aliquoted and stored at -20°C until further use. A 1.5 liter bacterial culture typically yielded about 20 mg of purified target protein.

Cell Culture

N18-RE-105 neuronal hybrid cells were cultured as previously described with minor modifications (Malouf et al., *J. Biol. Chem.* 1984, **259:**12756-12762). Cells were maintained in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 10% fetal bovine serum (Gibco), 1x HAT media supplement (Sigma), and antibiotic/antimycotic (Sigma).

HUT 102/6TG cells were maintained in RPMI 1640 medium (Gibco) supplemented with 10% fetal bovine serum (Hyclone), 2 mM glutamine, 50 IU/ml penicillin, and 50 µg/ml streptomycin (Gazdar et al., *Blood* 1980, **55:**409-417; Waldmann, *Adv. Exp. Med. Biol.* 1987, **213:**129-137).

Primary dissociated cultures from E17-E18 rat striatum were prepared as described with the exception that cells were placed in serum-free, defined media four hours after plating to curb proliferation of glial cells (Konradi et al., *J. Neurosci.*, 1994, **14:**5623-5634). The defined media consisted of DMEM/F12 media (Gibco) containing 0.45% glucose, B-27 supplement (Gibco), and antibiotics. Cells were plated at a density of 1.5×10^6 cells/2 ml/well in 6-well plates (Costar) precoated with 0.2% polyethylenimine.

Cytotoxicity Assay

Since the cytotoxicity of DTx is known to be mediated by its ability to inhibit cellular protein synthesis, we chose to examine the effect of our fusion toxins on cell viability through the use of a [^{14}C]-leucine incorporation assay.

[^{14}C]-Leucine incorporation into HUT 102/6TG cells and N18-RE-105 cells was measured using previously established methods (vanderSpek et al., *J. Biol. Chem.* 1996, **271:**12145-12149). For N18-RE-105 cultures, 1×10^5 cells in 0.1 ml of complete medium were seeded into each well of a flat bottom, 96-well microtiter plate. Assays conducted with HUT 102/6TG cells used 5×10^4 cells/well. In general, test proteins were serially diluted in

medium such that the addition of 0.1 ml to each well resulted in a final concentration ranging from 100 nM to 100 fM. Cultures were then incubated for 18 hr at 37°C in a 5% CO_2/95% air atmosphere. The medium was subsequently removed and replaced with 0.2 ml of leucine-free minimal essential medium (Gibco) containing 1 µCi/ml [^{14}C]-leucine (0.250 mCi/mmole; Dupont/NEN), 2 mM glutamine, and antibiotics. After 2 hours, the medium was again removed and the cells were lysed by treatment with 60 µl/well 0.4 M KOH for 10 minutes at room temperature. Proteins were precipitated by the addition of 140 µl/well 10% trichloroacetic acid followed by another 10 min incubation at room temperature. Insoluble protein was collected on glass fiber filters using a PhD cell harvester (Cambridge Technology, Inc., Watertown, MA). Sample radioactivity was determined according to standard methods.

[^{14}C]-Leucine incorporation into the primary neuron cultures was similarly determined by adjusting for the increased volumes required for the larger culture wells. Cells were used for the cytotoxicity assays after 4-5 days *in vitro* when the neurons were incubated with test proteins for 18 hr in a 1 ml volume of fresh media. Cells were subsequently pulsed for 2 hr with 1 µCi [^{14}C]-leucine in a total volume of 1 ml minimal essential media. After lysing the cells with 0.25 ml of KOH, proteins were precipitated by the addition of 0.6 ml of 10% trichloroacetic acid. Insoluble protein was collected on glass fiber filters and radioactivity was measured as above. Calculation of IC_{50} values was performed by determining the concentration of fusion toxin required to cause 50% inhibition of [^{14}C]-leucine incorporation following incubation of the cells with the toxin for 18 hr.

When indicated, statistical analysis of treatment groups was performed using one-way ANOVA followed by Tukey's test.

Results

Expression of the DAB_{389}TTC and DA(E149S)B389TTC fusion proteins in *E. coli* BL21(DE3) host cells was initially examined by Coomassie Blue staining of SDS-PAGE gels. Compared to total cell lysates obtained from parallel cultures of DAB389TTC and DA(E149S)B389TTC bacteria that were not treated with IPTG, the respective lysates obtained from IPTG-induced cultures each revealed a prominent band with an estimated molecular weight of 93,000. This electrophoretic mobility is in good accord with the size predicted for each of these 840 amino acid polypeptides. The DA(E149S)B_{389}TTC fusion

protein appeared to be present in a substantially greater amount than $DAB_{389}TTC$. SDS-PAGE analysis of soluble and insoluble protein fractions prepared from total lysates indicated that the $DAB_{389}TTC$ and $DA(E149S)B_{389}TTC$ target proteins were present in both fractions (data for E149S mutation not shown). The fusion proteins were subsequently purified from soluble bacterial protein using ammonium sulfate precipitation and ion exchange chromatography. Analysis of the purified target proteins by SDS-PAGE/Coomassie Blue staining indicated that the samples were approximately 85-90% pure. Western blot analysis of purified $DAB_{389}TTC$ protein with anti-TTC and anti-DTx A fragment antibodies confirmed the antigenic identity of the fusion protein; a major immunoreactive band corresponding to a MW of 93,000 was recognized by both antibodies. The presence of this immunopositive band in the soluble protein fraction obtained from a control culture indicates that there is leaky expression of $DAB_{389}TTC$ in the absence of IPTG.

We studied the functional properties of the $DAB_{389}TTC$ fusion protein through its ability to inhibit protein synthesis in N18-RE-105 neuronal hybrid cells and cultured embryonic rat striatal neurons. The N18-RE-105 cell line was chosen because, unlike most neuroblastoma cell lines, N18-RE-105 cells have a surface ganglioside composition similar to normal brain tissue and thus bind high amounts of tetanus toxin (Rogers and Snyder, *J. Biol. Chem.* 1981, **256**:2402-2407; Staub et al., *J. Neurosci.* 1986, **6**:1443-1451). This cell line has also been previously used to study the binding and internalization of another TTC fusion protein, SOD:Tet451 (Francis et al., *J. Biol. Chem.* 1995, **270**:15434-15442). Since N18-RE-105 cells have certain characteristics that are not typical of mammalian nerve cells, however, we further evaluated the activity of $DAB_{389}TTC$ in primary dissociated cultures of rat striatal neurons. Striatal neurons are not the natural target cells of TTx *in vivo* although the high density of TTx binding sites in rat striatum (Rogers and Snyder, *infra.*) indicated that these cells would nonetheless be useful for evaluating the interaction of the chimeric toxin with primary neurons *in vitro*.

Following overnight treatment of cultured striatal neurons or N18-RE-105 cells with various concentrations of $DAB_{389}TTC$, the chimeric toxin was shown to be a potent inhibitor of cellular protein synthesis. The inhibition of cellular [^{14}C]-leucine incorporation by $DAB_{389}TTC$ was dose-dependent and resulted in an IC_{50} of 4 pM in the primary neuron cultures and 2 nM in cultures of N18-RE-105 cells (Figure 1). The cytotoxic effect of $DAB_{389}TTC$ on cultured cells appeared to be specific for neuronal-type cells given that the chimeric toxin at a concentration of 100 nM produced only a modest 23% inhibition of

protein synthesis in HUT 102/6TG cells, a human T lymphocyte cell line known to have a large number of interleukin-2 (IL-2) receptors (Gazdar et al., *Blood* 1980, **55**:409-417; Waldmann, *Adv. Exp. Med. Biol.* 1987, **213**:129-137). When HUT 102/6TG cells were treated overnight with the DTx:IL-2 fusion toxin, DAB_{389}IL-2, however, incorporation of [^{14}C]-leucine was markedly reduced (IC_{50} = 10 pM,). We also studied the targeting specificity of DAB389TTC by comparing its cytotoxic potency in primary neurons to that of native DTx and another DTx fusion protein having a different cell targeting domain, DAB_{389}MSH (Murphy et al., *Proc. Natl. Acad. Sci. USA* 1986, **83**:8258-8261). As shown in Figure 2, the potency of DAB_{389}TTC for inhibiting [^{14}C]-leucine incorporation in cultured striatal neurons was generally 1000-fold greater than that observed with native DTx or DAB_{389}MSH.

To ascertain that DAB_{389}TTC cytotoxicity in cultured striatal neurons was mediated by the TTC cell targeting domain of the chimeric toxin, we first compared the cytotoxicity of DAB_{389}TTC alone with that of DAB_{389}TTC and tetanus antitoxin together. Simultaneous incubation of cultured striatal neurons with 10 pM DAB_{389}TTC and tetanus antitoxin almost completely prevented the inhibition of [^{14}C]-leucine incorporation observed after treatment with DAB_{389}TTC by itself (Figure 3). The binding profile of the chimeric toxin was further assessed by testing whether a large molar excess of purified recombinant TTC could prevent the cytotoxicity of DAB_{389}TTC in primary neurons. The chimeric toxin was also shown to inhibit [^{14}C]-leucine incorporation in primary neurons was only partially blocked by a 10,000-fold higher concentration of recombinant TTC.

Following receptor-mediated endocytosis of DTx into cellular endosomes, delivery of the DTx catalytic domain through the endosomal membrane into the cytosol is dependent upon acidification of endocytic vesicles. Agents that interfere with endosomal acidification, such as chloroquine, monensin, and bafilomycin A, are well known to diminish the cytotoxicity of DTx and DTx fusion proteins (Draper and Simon, *J. Cell Biol.* 1980, **87**:849-854; Fisher et al., *Proc. Natl. Acad. Sci., USA* 1996, **93**:7341-7345; Papini et al., *J. Biol. Chem.* 1993, **268**:1567-1574; Sandvig and Olsnes, *J. Cell Biol.* 1980, **87**:828-832). To determine if inhibition of protein synthesis in DAB_{389}TTC treated cells is likewise dependent on endosomal acidification, N18-RE-105 neuronal hybrid cells were coincubated with the chimeric toxin and either chloroquine or monensin. Both chloroquine and monensin substantially reversed the cytotoxic effect of DAB389TTC in N18-RE-105 cells.

Finally, to demonstrate that inhibition of protein synthesis associated with $DAB_{389}TTC$ treatment of neuronal cells is mediated through the ADP ribosylation activity of the DTx catalytic domain, we compared the cytotoxicity of DAB389TTC with that of an ADP ribosyltransferase defective mutant, $DA(E149S)B_{389}TTC$ (Barbieri and Collier, *Infect. Immun.* 1987, **55**:1647-1651; Wilson et al., *Biochem.* 1990, **29**:8643-8651). Consistent with previous results obtained in other cells treated with DTx and other DTx-related fusion proteins bearing this same mutation (Fisher et al., *Proc. Natl. Acad. Sci., USA* 1996, **93**:7341-7345; Lemichez et al., *Mol. Microbiol.* 1997, **23**:445-457; vanderSpek et al., *J. Biol. Chem.* 1996, **271**:12145-12149), $DA(E149S)B_{389}TTC$ was seen to be almost 1000-fold less potent than $DAB_{389}TTC$ in inhibiting [^{14}C]-leucine incorporation into cultured striatal neurons. The reduction in cytotoxicity associated with the E149S mutant was also observed in the N18-RE-105 cell line. Whereas the IC_{50} for $DAB_{389}TTC$ inhibition of protein synthesis in N18-RE-105 cells was 2 nM, the E149S mutant was essentially inactive at the highest concentration tested (100 nM).

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Equivalents include minor modifications to the domains described above whereby the modified domain retains the desired activity (or even has enhanced such activity), such as can be prepared and screened by conventional methodologies. Such equivalents are intended to be encompassed by the following claims.

All references disclosed herein are incorporated by reference in their entirety.

We claim:

Claims

1. A hybrid protein comprising:

(a) a catalytic domain moiety of diphtheria toxin that retains its cytotoxic activity following uptake of the hybrid protein into a neuronal cell;

(b) a membrane translocation domain moiety of diphtheria toxin that facilitates uptake of the hybrid protein into a neuronal cell; and

(c) a neuronal cell-binding ligand that selectively delivers the hybrid protein to neuronal cells.

2. The hybrid protein of claim 1, wherein the neuronal cell-binding ligand that selcctively delivers the hybrid protein to neuronal cells is tetanus toxin.

3. The hybrid protein of claim 1, wherein the tetanus toxin is a tetanus toxin fragment C moiety.

4. The hybrid protein of claim 1, wherein the catalytic domain moiety of diphtheria toxin contains mutations that enhance its cytotoxicity following uptake of the hybrid protein into a neuronal cell

5. The hybrid protein of claim 1, wherein the catalytic domain moiety of diphtheria toxin contains mutations that its cytotoxicity following uptake of the hybrid protein into a neuronal cell.

6. A hybrid protein comprising:

(a) a catalytic domain moiety of diphtheria toxin containing mutations that abolish its cytotoxic activity following uptake of the hybrid protein into a neuronal cell;

(b) a membrane translocation domain moiety of diphtheria toxin that facilitates uptake of the hybrid protein into a neuronal cell; and

(c) a neuronal cell-binding domain that selectively targets the hybrid protein to neuronal cells.

7. The hybrid protein of claim 6, further comprising a cytotoxic moiety, other that the diphtheria catalytic domain moiety, joined to the mutated diphtheria catalytic domain moiety by at least one covalent bond.

8. A hybrid protein comprising:

(a) a cytotoxic moiety, other than a diphtheria toxin catalytic domain moiety;

(b) a membrane translocation domain moiety of diphtheria toxin that facilitates uptake of the hybrid protein into a neuronal cell; and

(c) a neuronal cell-binding domain that selectively targets the hybrid protein to neuronal cells.

9. A hybrid protein comprising:

(a) a cytotoxic moiety, other than a diphtheria toxin catalytic domain moiety;

(b) a membrane translocation domain moiety of a protein; and

(c) a neuronal cell-binding domain that selectively targets the hybrid protein to neuronal cells.

10. The hybrid protein of claim 9, wherein the membrane translocation domain moiety of a protein is the translocation domain moiety of a naturally occurring toxin that facilitates uptake of the hybrid protein into a neuronal cell.

11. The hybrid protein of claim 10, wherein the naturally occurring toxin is a toxin selected from the group consisting of diphtheria toxin and Pseudomonas exotoxin A.

12. A hybrid protein comprising:

(a) a cytotoxic agent; and

(b) a neuronal cell-binding domain that selectively targets the hybrid protein to neuronal cells.

13. The hybrid protein of claim 12, wherein the neuronal cell-binding domain that selectively targets the hybrid protein to neuronal cells is a nerve-cell binding fragment of tetanus toxin.

14. A pharmaceutical composition comprising:

a hybrid protein according to anyone of claims 1-13, in a pharmaceutically effective amount to inhibit protein synthesis in a neuronal cell leading to neuronal cell death, and

a pharmaceutically acceptable carrier.

15. A method of inhibiting protein synthesis in a neuronal cell, comprising:

contacting a neuronal cell with a hybrid protein of claim 1, in an effective amount to inhibit protein synthesis in the neuronal cell.

16. A method of treating spasm or involuntary contraction in a muscle or a group of muscles in a subject, comprising:

administering to a muscle or a group of muscles in a subject in need of such treatment a hybrid protein according to any one of claims 1-13, in an effective amount to enhance denervation of the muscle or group of muscles and inhibit spasm or involuntary contraction in the muscle or the group of muscles of the subject.

17. The method of claim 16, wherein the subject in need of such treatment has blepharospasm, strabismus, spasmodic torticollis, focal dystonia, jaw dystonia, occupational dystonia, corneal ulceration, spasmodic dysphonia, or facial dyskinesis.

18. The method of claim 17, wherein the facial dyskinesis is Meige syndrome, hemifacial spasm, aberrant regeneration of facial nerves, or apraxia of eyelid opening.

19. A method of treating spasm or involuntary contraction in a muscle or a group of muscles in a subject, comprising:

administering to a muscle or a group of muscles in a subject in need of such treatment a pharmaceutical composition of claim 14, in an effective amount to enhance denervation of the muscle or group of muscles and inhibit spasm or involuntary contraction in the muscle or the group of muscles of the subject.

20. A method of enhancing relaxation or slackening of cutaneous tissue, comprising:

locally administering to a cutaneous tissue a hybrid protein according to any one of claims 1-13, in an effective amount to enhance denervation of the muscle or group of

muscles present subcutaneously to the cutaneous tissue to enhance relaxation or slackening of the cutaneous tissue.

21. The method of claim 20, wherein the hybrid protein according to any one of claims 1-13 is applied in a composition in an amount ranging from 0.00001 to 20% by weight, based on the total weight of the composition.

22. The method of claim 21, wherein the hybrid protein according to any one of claims 1-13 is applied in a composition in an amount ranging from 0.01 to 10% by weight, based on the total weight of the composition.

23. The method of claim 20, further comprising co-administering at least one of a hydroxy acid and a retinoid.

24. The method of claim 23, wherein the hydroxy acid is selected from the group consisting of α-hydroxy acids and β-hydroxy acids, which can be linear, branched or cyclic and saturated or unsaturated.

25. The method of claim 23, wherein the retinoid is selected from the group consisting of retinoic acid, retinol and retinol esters.

26. A method for lessening wrinkles or fine lines, by relaxing or slackening cutaneous tissue, comprising:

locally administering to a cutaneous tissue a hybrid protein according to any one of claims 1-13 in an effective amount to enhance denervation of the muscle or group of muscles present subcutaneously to the cutaneous tissue to enhance relaxation or slackening of the cutaneous tissue lessening wrinkles or fine lines.

27. A method of controlling autonomic nerve function in a subject, comprising:

locally administering to a target tissue or organ of a subject in need of such treatment a hybrid protein according to any one of claims 1-13, in an effective amount to enhance denervation in the target tissue or organ and control autonomic nerve function in the subject.

28. The method of claim 27, wherein the autonomic nerve function includes the function of an autonomic nerve which contributes to at least one symptom of rhinorrhea, otitis media, excessive salivation, asthma, COPD, excessive stomach acid secretion, spastic colitis or excessive sweating.

29. A method of treating a neurodegenerative disorder in a subject, comprising:

locally administering to a target area in the brain of a subject in need of such treatment a pharmaceutical composition of claim 14, in an effective amount to enhance neuronal cell-death in the target area of the brain of the subject.

30. A method of treating a neurodegenerative disorder in a subject, comprising:

locally administering to a target area in the brain of a subject in need of such treatment a hybrid protein according to any one of claims 1-13, in an effective amount to enhance neuronal cell-death in the target area of the brain of the subject.

31. The method of claim 27, wherein the subject has a neurodegenerative disorder selected from the group consisting of amyotrophic lateral sclerosis, Parkinson's disease, Huntington's disease, Alzheimer's disease, multiple sclerosis, olivopontocerebellar atrophy, multiple system atrophy, progressive supranuclear palsy, diffuse Lewy body disease, corticodentatonigral degeneration, progressive familial myoclonic epilepsy, strionigral degeneration, torsion dystonia, familial tremor, Down's Syndrome, Gilles de la Tourette syndrome, Hallervorden-Spatz disease, diabetic peripheral neuropathy, dementia pugilistica, AIDS Dementia, age related dementia, age associated memory impairment, amyloidosis-related neurodegenerative diseases, traumatic brain injury, cerebral edema, schizophrenia, peripheral nerve damage, spinal cord injury, Wernicke-Korsakoff's related dementia.

32. The method of claim 27, wherein the subject has Parkinson's disease.

FIGURE 1.

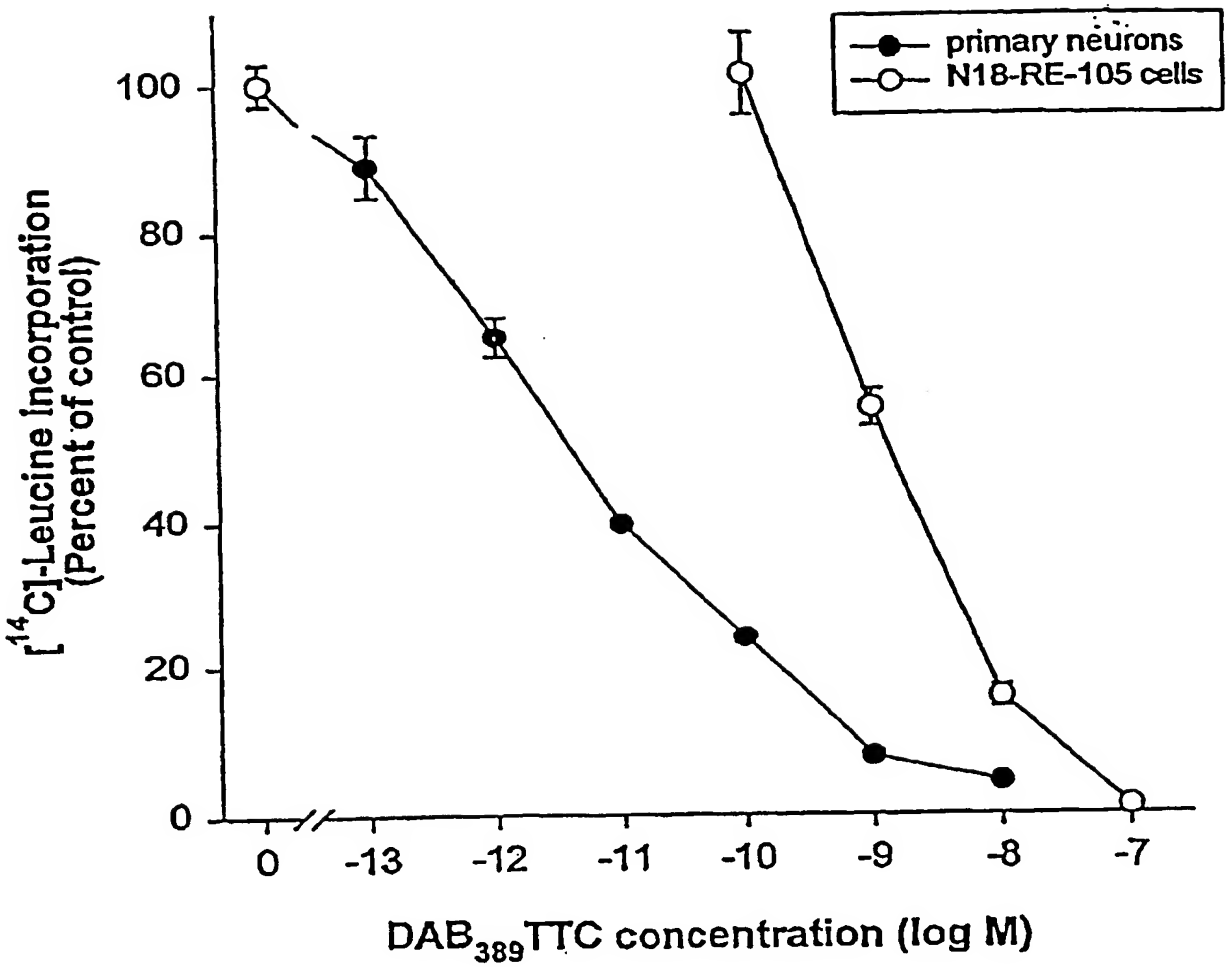


FIGURE 2.

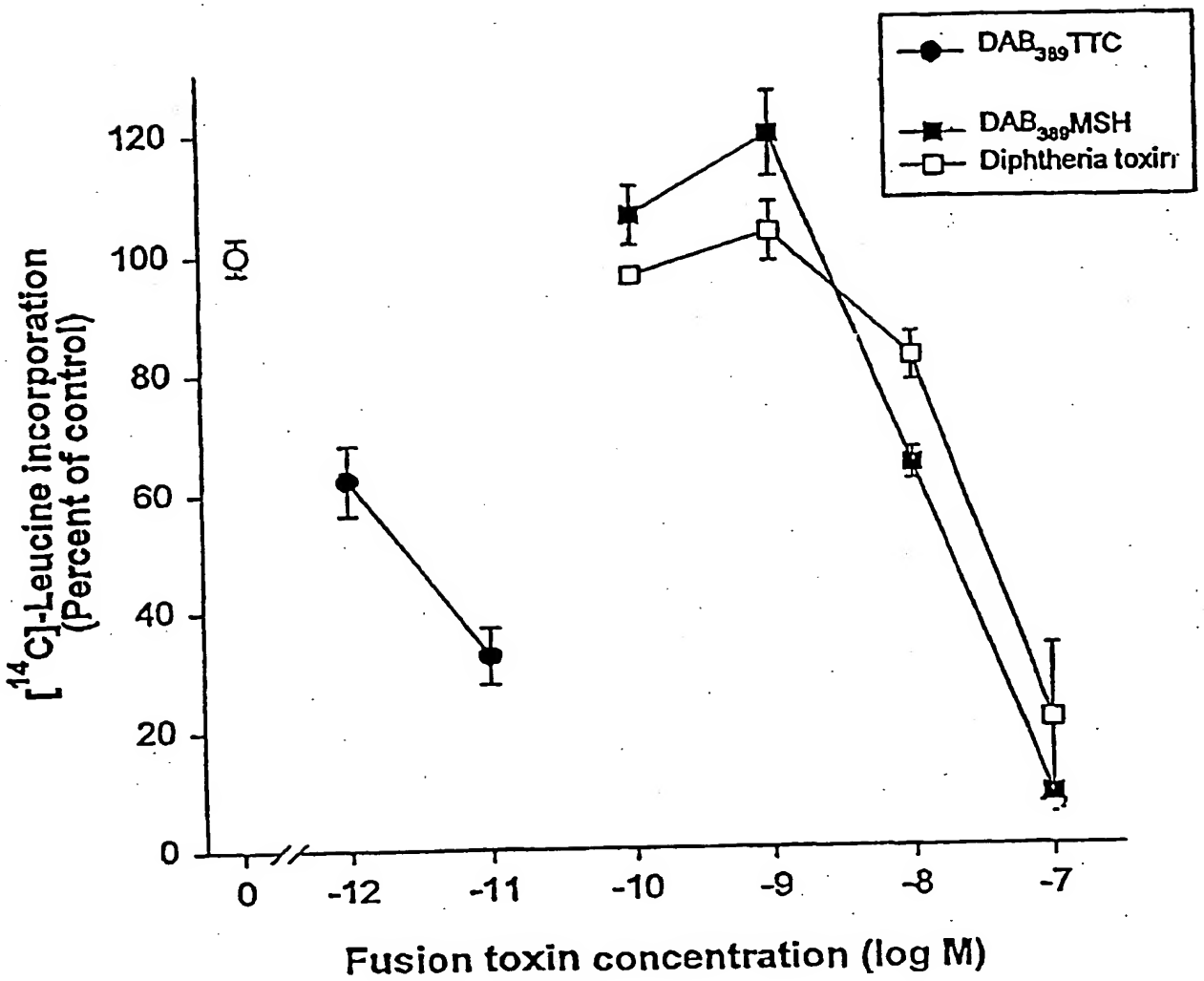

DAB389TTC
DAB389MSH
Diphtheria toxin
[14C]-Leucine incorporation (Percent of control)
120
100
80
60
40
20
0
0
-12
-11
-10
-9
-8
-7
Fusion toxin concentration (log M)

FIGURE 3.

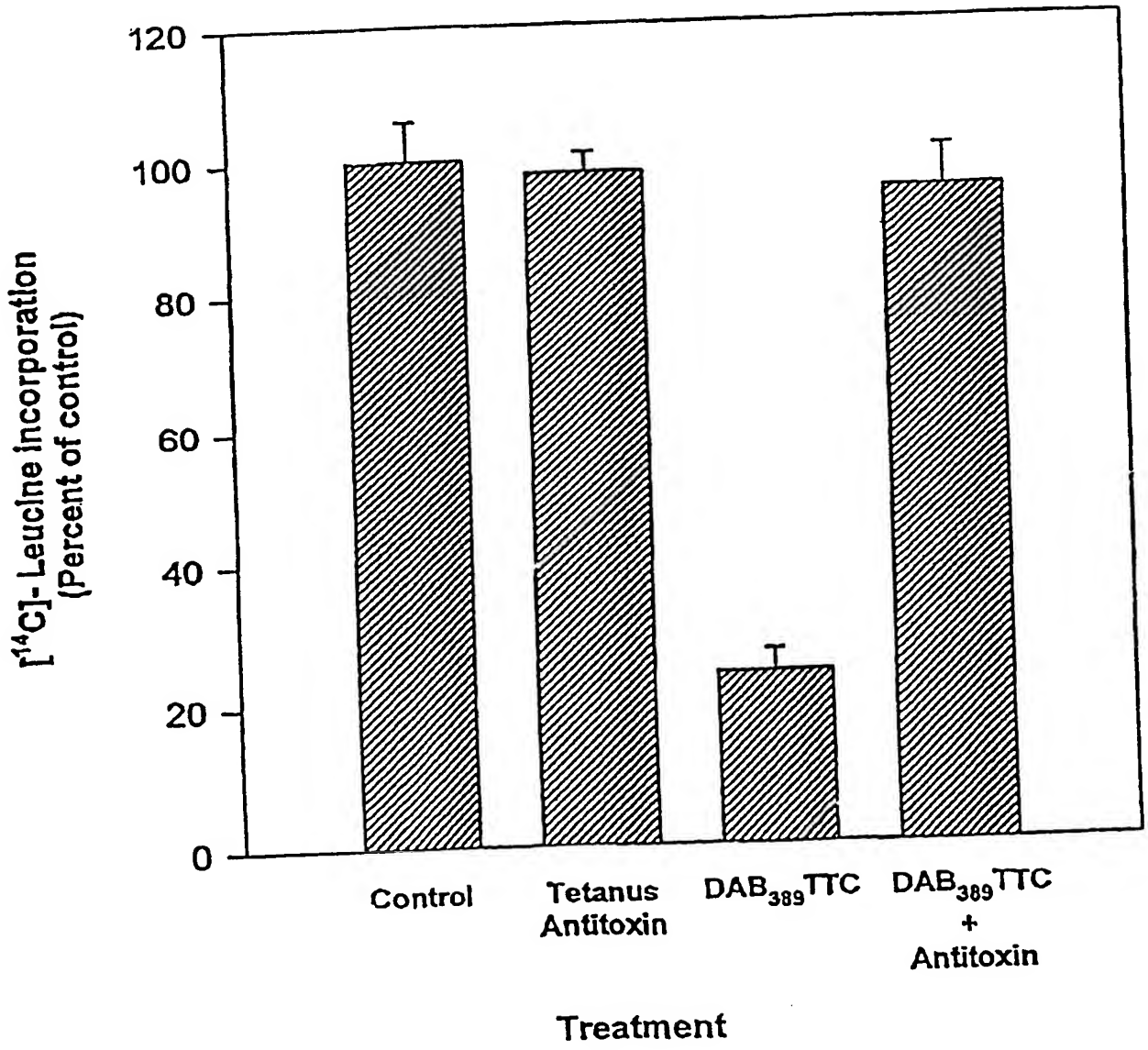

120
100
80
60
40
20
0
[14C]- Leucine incorporation
(Percent of control)
Control
Tetanus Antitoxin
DAB389TTC
DAB389TTC + Antitoxin
Treatment

FIG. 4A

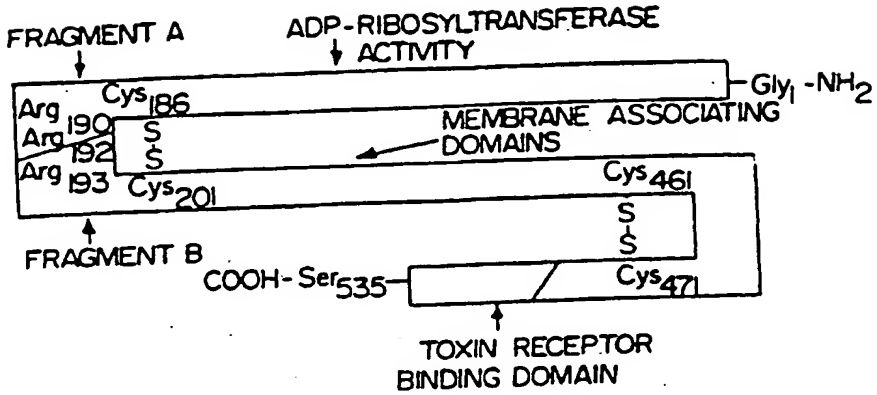
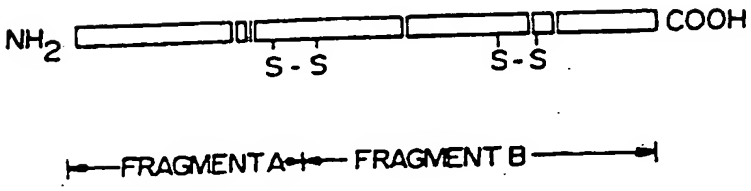


FIG. 4B



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Published:
— *with international search report*

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22 November 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/36588 A3

(54) Title: FUSION PROTEINS THAT SPECIFICALLY INHIBIT PROTEIN SYNTHESIS IN NEURONAL CELLS

(57) Abstract: This invention relates to compositions and methods for the specific inhibition of protein synthesis in neuronal cells leading to neuronal cell death. More specifically, the invention relates to hybrid protein molecules that show high specificity for, and increased cytotoxicity in, neuronal cells. Such hybrid molecules are useful in a variety of conditions where localized inhibition of neuronal cell function is desirable.

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A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : C07K 1/00, 2/00, 14/00; C12N 5/00; C12Q 1/00; G01N 33/53
US CL : 435/4, 7.1, 325; 530/300, 350

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/4, 7.1, 325; 530/300, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ROSSETTO et al. Killing of Neuroblastoma and Pheochromocytoma Cells in Colture with Tetanus-Diphtheria Hybrid Toxins. Eur. J. Cancer. Vol. 27, Supp 3, S84, Abstract No. 11.084, see entire abstract.	1-5, 14-15
Y --- A	GOMEZ-DUARTE et al. Expression of Fragment C of Tetanus Toxin Fused to a Carboxyl-Terminal Fragment of Diphtheria Toxin in Salmonella typhi CVD 908 Vaccine Strain. Vaccine. 1995, Vol. 13, No. 16, pages 1596-1602, see entire document.	1-3 --- 4-5, 14-15
Y --- A	COEN et al. Construction of Hybrid Proteins that Migrate Retrogradely and Transynaptically into the Central Nervous System. Proc. Natl. Acad. Sci. USA. August 1997, Vol 94, pages 9400-9405, see entire document.	1-3 --- 4-5, 14-15
A	US 5,780,024 A (BROWN et al) 14 July 1998 (14.07.1998), see entire document	1-5, 14-15
A.P	FRANCIS et al. Enhancement of Diphtheria Toxin Potency by Replacement of the Receptor Binding Domain with Tetanus Toxin C-Fragment: A Potential Vector for Delivering Heterologous Proteins to Neurons. J. Neurochem. 2000, Vol. 74, No. , pages 2528-2536, see entire document.	1-5, 14-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
05 June 2001 (05.06.2001)	13 JUL 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer BRIDGET E. BUNNER Telephone No. (703) 308-0196

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Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claim Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
Please See Continuation Sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: 1-5, 14-15, in part, drawn to a hybrid diphtheria-tetanus toxin protein that enhances cytotoxicity.

Remark on Protest
☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

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BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claim(s) 1-5, 14-15, in part, drawn to a hybrid protein comprising domain moieties of diphtheria toxin and tetanus toxin wherein the catalytic domain of diphtheria toxin contains mutations that retain or enhance cytotoxicity; a method of inhibiting protein synthesis in a neuronal cell.

Group II, claim(s) 6-7 and 14, in part, drawn to a hybrid protein comprising domain moieties of diphtheria toxin and tetanus toxin wherein the catalytic domain of diphtheria toxin contains mutations that abolish cytotoxicity.

Group III, claim(s) 8-11 and 14, in part, drawn to a hybrid protein comprising a cytotoxic moiety other than a diphtheria toxin, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain.

Group IV, claim(s) 12-14, in part, drawn to a hybrid protein comprising a cytotoxic agent and a neuronal cell-binding domain of tetanus toxin.

Group V, claim(s) 16-19, in part, drawn to a method of treating spasm or involuntary contraction in a muscle or group of muscles comprising administering a tetanus/tetanus toxin hybrid protein wherein the diphtheria toxin contains mutations that retain or enhance cytotoxicity.

Group VI, claim(s), 16-19, in part, drawn to a method of treating spasm or involuntary contraction in a muscle or group of muscles comprising administering a diphtheria/tetanus toxin hybrid protein wherein the diphtheria toxin contains mutations that abolish cytotoxicity.

Group VII, claims(s) 16-19, in part, drawn to a method of treating spasm or involuntary contraction in a muscle or group of muscles comprising administering a hybrid protein wherein the protein comprises a cytotoxic moiety other than diphtheria, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain.

Group VIII, claim(s) 16-19, in part, drawn to a method of treating spasm or involuntary contraction in a muscle or group of muscles comprising administering a hybrid protein wherein the protein comprises a cytotoxic agent and tetanus toxin neuronal cell-binding domain.

Group IX, claim(s) 20-26, in part, drawn to a method of enhancing relaxation or slackening of cutaneous tissue comprising locally administering to a cutaneous tissue a diphtheria/tetanus toxin hybrid protein wherein the diphtheria toxin contains mutations that retain or enhance cytotoxicity.

Group X, claim(s) 20-26, in part, drawn to a method of enhancing relaxation or slackening of cutaneous tissue comprising locally administering to a cutaneous tissue a diphtheria/tetanus toxin hybrid protein wherein the diphtheria toxin contains mutations that abolish cytotoxicity.

Group XI, claim(s) 20-26, in part, drawn to a method of enhancing relaxation or slackening of coetaneous tissue comprising locally administering to a cutaneous tissue a hybrid protein wherein the protein comprises a cytotoxic moiety other than diphtheria, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain.

Group XII, claim(s) 20-26, in part, drawn to a method of enhancing relaxation or slackening of cutaneous tissue comprising locally administering to a cutaneous tissue a hybrid protein wherein the protein comprises a cytotoxic agent and tetanus toxin neuronal cell-binding domain.

Group XIII, claim(s) 27-28 and 31-32, in part, drawn to a method of controlling autonomic nerve function comprising locally administering to a target tissue or organ a diphtheria/tetanus toxin hybrid protein in an amount effective to enhance denervation in the

target tissue or organ and control autonomic nerve function wherein the diphtheria toxin contains mutations that retain or enhance cytotoxicity.

Group XIV, claim(s) 27-28 and 31-32, in part, drawn to a method of controlling autonomic nerve function comprising locally administering to a target tissue or organ a tetanus/tetanus hybrid protein in an amount effective to enhance denervation in the target tissue or organ and control autonomic nerve function wherein the diphtheria toxin contains mutations that abolish cytotoxicity.

Group XV, claim(s) 27-28 and 31-32, in part, drawn to a method of controlling autonomic nerve function comprising locally administering to a target tissue or organ a hybrid protein in an amount effective to enhance denervation in the target tissue or organ and control autonomic nerve function wherein the protein comprises a cytotoxic moiety other than diphtheria, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain.

Group XVI, claim(s) 27-28 and 31-32, in part, drawn to a method of controlling autonomic nerve function comprising locally administering to a target tissue or organ a hybrid protein in an amount effective to enhance denervation in the target tissue or organ and control autonomic nerve function wherein the protein comprises a cytotoxic agent and tetanus toxin neuronal cell-binding domain.

Group XVII, claim(s) 29-30, in part, drawn to a method of treating a neurodegenerative disorder comprising locally administering to a target area in the brain a diphtheria/tetanus hybrid protein to enhance neuronal cell-death wherein the diphtheria toxin contains mutations that retain or enhance cytotoxicity.

Group XVIII, claim(s) 29-30, in part, drawn to a method of treating a neurodegenerative disorder comprising locally administering to a target area in the brain a hybrid protein to enhance neuronal cell-death wherein the diphtheria toxin contains mutations that abolish cytotoxicity.

Group XIX, claim(s) 29-30, in part, drawn to a method of treating a neurodegenerative disorder comprising locally administering to a target area in the brain a hybrid protein to enhance neuronal cell-death wherein the protein comprises a cytotoxic moiety other than diphtheria, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain.

Group XX, claim(s) 29-30, in part, drawn to a method of treating a neurodegenerative disorder comprising locally administering to a target area in the brain a hybrid protein to enhance neuronal cell-death wherein the protein comprises a cytotoxic agent and tetanus toxin neuronal cell-binding domain.

The inventions listed as Groups I-XX do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Groups I-IV claim different products which are structurally and functionally unrelated, each to each other. Group I recites the special technical feature of a hybrid protein comprising a catalytic domain of diphtheria toxin that enhances cytoxicity, a membrane translocation domain of diphtheria toxin, and a nerve cell binding fragment of tetanus toxin which is not required for the products of Groups II-IV. Group II recites the special technical feature of a hybrid protein comprising a catalytic domain of diphtheria toxin that reduces cytoxicity, a membrane translocation domain of diphtheria toxin, and a nerve cell binding fragment of tetanus toxin which is not required for the products of Groups I and III-IV. Group III recites the special technical feature of a hybrid protein comprising a cytotoxic moiety other than the catalytic domain of diphtheria toxin, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain which is not required for the products of Groups I-II and IV. Group IV recites the special technical feature of a hybrid protein comprising a cytotoxic agent and a nerve cell binding fragment of tetanus toxin which is not required by the products of Groups I-III.

Groups V-XX claim different methods. Group V recites the special technical feature of treatment of spasm or involuntary contraction in a muscle or a group of muscles and administration of a diphtheria/tetanus hybrid protein wherein the protein toxin contains mutations that retain or enhance cytotoxicity which is not required by the methods of Groups VI-XX. Group VI recites the special technical feature of treatment of spasm or involuntary contraction in a muscle or a group of muscles and administration of a diphtheria/tetanus hybrid protein wherein the protein toxin contains mutations that abolish cytotoxicity which is not required by the methods of Groups V and VII-XX. Group VII recites the special technical feature of treatment of spasm or involuntary contraction in a muscle or a group of muscles and administration of a hybrid protein wherein the protein comprises a cytotoxic moiety other than diphtheria, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain which is not required by the methods of Groups V-VI and VIII-XX. Group VIII recites the special technical feature of treatment of spasm or involuntary contraction in a muscle or a group of muscles and administration of a hybrid protein wherein the protein comprises a cytotoxic agent and tetanus toxin domain which is not required by the methods of V-VII and IX-XX. Group IX recites the special technical feature of relaxation or slackening of cutaneous tissue and administration of a diphtheria/tetanus hybrid protein wherein the protein toxin contains mutations that retain or enhance cytotoxicity which is not required by the methods of Groups VI-VIII and X-XX. Group X recites the special technical feature of relaxation or slackening of cutaneous tissue and administration of a diphtheria/tetanus hybrid protein wherein the protein toxin contains mutations that abolish cytotoxicity which is not required by the methods of Groups VI-IX and XI-XX. Group XI recites the special technical feature of relaxation or slackening of cutaneous tissue

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and administration of a hybrid protein wherein the protein comprises a cytotoxic moiety other than diphtheria, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain which is not required by the methods of Groups V-X and XII-XX. Group XII recites the special technical feature of relaxation or slackening of cutaneous tissue and administration of a hybrid protein wherein the protein comprises a cytotoxic agent and tetanus toxin domain which is not required by the methods of V-XI and XIII-XX. Group XIII recites the special technical feature of enhancement of denervation in target tissue or organ and control of autonomic nerve function and administration of a diphtheria/tetanus hybrid protein wherein the protein toxin contains mutations that retain or enhance cytotoxicity which is not required by the methods of Groups V-XII and XIV-XX. Group XIV recites the special technical feature of enhancement of denervation in target tissue or organ and control of autonomic nerve function and administration of a diphtheria/tetanus hybrid protein wherein the protein toxin contains mutations that abolish cytotoxicity which is not required by the methods of Groups V-XIII and XV-XX. Group XV recites the special technical feature of enhancement of denervation in target tissue or organ and control of autonomic nerve function and administration of a hybrid protein wherein the protein comprises a cytotoxic moiety other than diphtheria, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain which is not required by the methods of Groups V-XIV and XVI-XX. Group XVI recites the special technical feature of enhancement of denervation in target tissue or organ and control of autonomic nerve function and administration of a hybrid protein wherein the protein comprises a cytotoxic agent and tetanus toxin domain which is not required by the methods of V-XV and XVII-XX. Group XVII recites the special technical feature of administration of a diphtheria/tetanus hybrid protein to a target area in the brain wherein the protein toxin contains mutations that retain or enhance cytotoxicity, which is not required by the methods of Groups VI-XVI and XVIII-XX. Group XVIII recites the special technical feature of administration of a diphtheria/tetanus hybrid protein to a target area in the brain wherein the protein toxin contains mutations that abolish cytotoxicity which is not required by the methods of Groups VI-XVII and XIX-XX. Group XIX recites the special technical feature of administration of a hybrid protein to a target area in the brain wherein the protein comprises a cytotoxic moiety other than diphtheria, a membrane translocation domain of diphtheria toxin or Pseudomonas exotoxin A, and a neuronal cell-binding domain which is not required by the methods of Groups V-XVIII and XX. Group XX recites the special technical feature of administration of a hybrid protein to a target area in the brain wherein the protein comprises a cytotoxic agent and tetanus toxin domain which is not required by the methods of Groups V-XIX. Also, the PCT rules do not provide for the search and examination of more than one method of use and one method of making for the first claimed product.

Continuation of B. FIELDS SEARCHED Item 3:
EAST, DIALOG, MEDLINE
search terms: authors' names, diphtheria, tetanus, neuro, brain, fusion, chimera, hybrid